Analytica Chimica Acta 852 (2014) 284–292

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Zirconium silicate assisted removal of residual proteins after organic solvent deproteinization of human plasma, enhancing the stability of the LC–ESI-MS response for the bioanalysis of small molecules

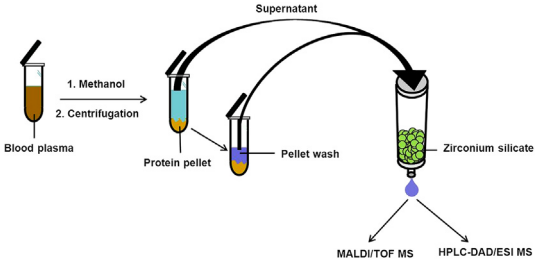
Shah Hussain [a], Cornelia Pezzei [a], Yüksel Güzel [a,b], Matthias Rainer [a], Christian W. Huck [a,*], Günther K. Bonn [a,b]

[a] Institute of Analytical Chemistry and Radiochemistry, CCB-Center for Chemistry and Biomedicine, Leopold-Franzens University, Innrain 80/82, 6020 Innsbruck, Austria
[b] ADSI-Austrian Drug Screening Institute, Innrain 66a, 6020 Innsbruck, Austria

HIGHLIGHTS

- A novel sample preparation technique for isolation of small molecules from human plasma.
- Effectiveness of zirconium silicate for the removal of residual proteins after protein precipitation.
- Abolishing the consumption of salts for the depletion of residual proteins after protein precipitation.
- More than 99.6% removal of plasma proteins.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 May 2014
Received in revised form 5 September 2014
Accepted 8 September 2014
Available online 16 September 2014

Keywords:
Solid-phase extraction
Zirconium silicate
Human plasma
Caffeoylqinic acids
Protein precipitation

ABSTRACT

An efficient blood plasma clean-up method was developed, where methanol protein precipitation was applied, followed by zirconium silicate assisted exclusion of residual proteins. A strong binding of zirconium (IV) silicate to the proteins enabled the elimination of remaining proteins after solvent deproteinization through a rapid solid-phase extraction (SPE) procedure. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF MS) was used for monitoring the proteins during clean-up practice applied to human plasma samples. The proteins were quantified by colorimetric detection using the bicinchoninic acid (BCA) assay. The presented analytical strategy resulted in the depletion of >99.6% proteins from human plasma samples. Furthermore, high-performance liquid chromatography hyphenated to diode-array and electrospray ionization mass spectrometric detection (HPLC–DAD/ESI MS) was applied for qualitative and quantitative analysis of the caffeoylquinic acids (CQAs) and their metabolites in human plasma. The procedure demonstrated high recoveries for the standard compounds spiked at different concentrations. Cynarin and chlorogenic acid

Abbreviations: ACN, acetonitrile; ALE, artichoke leaves extract; BCA, bicinchoninic acid; BDS, base deactivated silica; BSA, bovine serum albumin; CA, caffeic acid; CGA, chlorogenic acid; CQAs, caffeoylqinic acids; CV, co-efficient of variance; CYN, cynarin; DAD, diode-array detection; DL, desolvation line; DHCA, 3,4-dihydroxyhydrocinnamic acid; ESI, electrospray ionization; FA, formic acid; FLA, ferulic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LLE, liquid–liquid extraction; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; m/z, mass-to-charge; QC, quality control; RE, relative error; SA, sinapinic acid; SALLE, salting-out assisted liquid/liquid extraction; SEM, scanning electron microscopy; SIM, selected ion monitoring; S/N, signal to noise; SPE, solid-phase extraction; TFA, trifluoroacetic acid; TOF, time-of-flight; XIC, extracted ion chromatograms.

* Corresponding author at: Head of Spectroscopy Group, Institute of Analytical Chemistry and Radiochemistry, CCB-Center for Chemistry and Biomedicine, Leopold-Franzens University, Innrain 80/82, 6020 Innsbruck, Austria. Tel.: +43 512 507 57304, fax: +43 512 507 57399.
E-mail address: Christian.W.Huck@uibk.ac.at (C.W. Huck).

http://dx.doi.org/10.1016/j.aca.2014.09.014

were recovered in the range of 81–86% and 78–83%, respectively. Caffeic acid was extracted in the excess of 89–92%, while ferulic acid and dihydroxyhydrocinnamic acid showed a recovery of 87–91% and 92–95%, respectively. The method was partially validated in accordance with FDA-Industry Guidelines for Bioanalytical Method Validation (2001). The presented scheme improves the clean-up efficacy of the methanol deproteinization, significantly reduces the matrix effects and provides a great analytical tool for the isolation of small molecules from human plasma.

1. Introduction

Blood plasma contains about 10,000 different proteins with the total concentration of 6 to 8 g dL^{-1} [1]. The generally used sample preparation techniques for biological sample cleaning include protein precipitation, liquid-liquid extraction (LLE), SPE and ultrafiltration [2,3]. Each of these approaches is associated with certain advantages and disadvantages.

Protein precipitation is one of the earliest and the least time-consuming sample preparation technique for purifying biological fluids. It involves denaturation of proteins by external stress (such as strong acid/base, heat or organic solvents) and consequently disrupts protein–drug binding [4,5]. However, not all protein precipitation methods are suitable for removing the proteins for the analysis of small molecules. The protein precipitation techniques based upon salts, acids and heat usually trap the analytes in the protein aggregates leaving a small quantity in the supernatant. Therefore, nearly all bioanalytical protein precipitation methods use water miscible organic solvents for protein denaturation [2,6]. Most of the organic solvent protein precipitation methods achieve only about 90–96% protein precipitation from human plasma [7]. The remaining proteins still can interfere with the chromatographic procedures by generating matrix effects. Many approaches have been employed to overcome this problem by additional cleaning of the sample. In this regard, salting-out assisted liquid/liquid extraction technique (SALLE) has recently been introduced to further clean-up the biological samples for the subsequent liquid chromatography (LC). SALLE involves double protein precipitation i.e., conventional organic solvent deproteinization followed by the addition of salts into the supernatant, which results in the removal of more than 99% plasma proteins [8,9].

LLE provides cleaner samples and is applied in many studies, but it is quite time consuming due to slow drying of extraction solvents. Moreover, LLE is unsuitable for hydrophilic compounds [3]. SPE is easy to use and efficient, but rather expensive compared to protein precipitation. It further needs the selection of an appropriate affinity sorbent, method development, and optimization for isolating different classes of compounds from the biological samples [2]. Ultrafiltration is the least common sample preparation technique for the biological samples. It has the potential to deplete more than 99% of proteins from human plasma [10]; however, the ultrafiltrate only provides a measure of drug not bounded to proteins as opposed to total drug. Therefore, it is highly inappropriate for strongly protein-bound drugs. In addition, separate experiments must be executed to determine, and correct for the drug-membrane binding. The choice of membrane is certainly critical for the analysis as membrane contaminants and structure can affect the quantitation and hence results in the loss of recovery [11,12].

In this study, we combined methanol–protein precipitation and a follow-up cleaning using zirconium silicate powder in spin-columns. Artichoke leaves extract (ALE) tablets 400 mg were administrated orally for three days to a healthy volunteer for setting-up a practical application of the clean-up method for the isolation of CQAs and their metabolites from human plasma.

ALE is traditionally used for the treatment of hepatic and dyspeptic orders. Efficacy has been established in numerous clinical studies [13]. ALE is used for the treatment of the irritable-bowel syndrome [14] and reduces the risk of coronary heart disease by lowering plasma cholesterol levels [15]. Antioxidant activities [16] and the inhibition of hepatocellular cholesterol biosynthesis [17] were also observed. CQAs are one of the key active substances of ALE. CQAs are the esters of quinic acids with caffeic, ferulic or p-coumaric acids. Humans routinely ingest CQAs from various plants [18], fruits [19], coffee [20] and green tea [21]. There have been numerous reports on the pharmacological activities of CQAs [22–29]. CQAs (chlorogenic acid (CGA) and cynarin (CYN)) and three of their potential metabolites [30] (caffeic acid (CA), ferulic acid (FLA) and 3,4-dihydroxyhydrocinnamic acid (DHCA)) were selected as model compounds for the study.

The aim of this study was to design a rapid, convenient and an efficient clean-up method for human plasma samples. The presented analytical workflow should assist the popular organic solvent deproteinization as a powerful sample preparation tool. Furthermore, this novel method shows great potential to be established as an analytical platform for the isolation of small molecules from human plasma.

2. Materials and methods

2.1. Reagents

Acetonitrile (ACN, HPLC-grade), methanol (ultra LC–MS grade) and water (HPLC-grade) were purchased from Carl Roth Gmbh + Co., KG (Karlsruhe, Germany). Formic acid (FA) was obtained from Merck KGaA (Darmstadt, Germany). Sinapinic acid (SA), zirconium (IV) silicate (325 mesh), Supel™ QuE Z-Sep/C18 (sorbent amount 60 mg, centrifuge tube volume 2 mL), SigmaPrep™ spin columns (7–20 microns polyethylene frit, 800 μL sample capacity), bovine serum albumin (BSA) and human plasma (blank plasma), chlorogenic acid (CGA, 95%), ferulic acid (FLA, 99%), 3,4-dihydroxyhydrocinnamic acid (DHCA, 98%), were purchased from Sigma–Aldrich (St. Louis, USA). Cynarin (CYN) was from Extrasynthese (Genay, France), caffeic acid (CA, 97%), trifluoroacetic acid (TFA, for protein sequence analysis), gallic acid, were from Fluka (Buchs, Switzerland). The chemical structures of CGA, CYN, CA, FLA and DHCA are shown in Fig. 1A–E, respectively. BCA™ protein assay kit was from Pierce–Thermo Fisher Scientific (Rockford, Illinois, USA).

2.2. Instrumentation

2.2.1. MALDI-TOF-MS

For qualitative analysis of proteins, a Bruker Daltonics Ultraflex I MALDI-TOF/TOF instrument (Bremen, Germany) was used. 1 μL of the sample was spotted on a stainless steel target followed by 1 μL of saturated SA solution (SA in ACN/water (1/1, v/v) containing 0.1% TFA). All measurements were recorded in linear mode. Mass spectra were recorded by summing up 500 laser shots. Laser power was attenuated at 70% of its maximal intensity, using a 337 nm nitrogen laser at 50 Hz. The Flex Analysis version 2.4 software provided by the manufacturer was used for data processing.